In Vitro Lymphocyte Response to Purified Protein Derivative, BCG, and *Mycobacterium leprae* in a Population Not Exposed to Leprosy

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Lymphocytes from 14 BCG-vaccinated donors, seven tuberculin positive and seven tuberculin negative by skin testing, were stimulated in vitro with four mycobacterial antigens, purified protein derivative (PPD), PPD/BCG, whole BCG bacilli, and whole *Mycobacterium leprae* and also with *Candida* antigen and phytohemagglutinin. The response was measured by incorporation of \(^{3}H\)-labeled thymidine. The response to PPD, PPD/BCG, and BCG was found to correlate with the result of skin testing with tuberculin. The tuberculin-positive group also responded more strongly to *M. leprae*, whereas the two groups did not differ significantly in their response to *Candida* antigen or phytohemagglutinin. These findings indicate a certain degree of cross-reactivity between BCG and *M. leprae*. The use of the lymphocyte transformation test to measure antigenic cross-reactivity is discussed.

The clinical course of leprosy seems to be intimately related to the patient's cell-mediated immune response against *Mycobacterium leprae*. The early skin reaction to lepromin (Fernandez reaction) reflects the individual's delayed hypersensitivity, whereas the late reaction (Mitsuda reaction) may represent a primary immune response to the lepromin itself (28). A positive lepromin test does not necessarily indicate previous contact (29). BCG vaccination may induce lepromin positivity (7, 8), and exposure to other mycobacteria probably has a similar effect. Therefore, the lepromin test has not been very useful in diagnostic and epidemiological work in leprosy (21).

More recently, the lymphocyte transformation test (LTT) has been introduced to measure delayed hypersensitivity against *M. leprae* (6, 11, 13). Godal et al. (10) claimed that their LTT showed a high degree of specificity and thus could be used for epidemiological purposes. In a group of 18 persons who had not previously worked with leprosy, and of whom at least 12 had been BCG vaccinated, the average response to BCG was 7.03% transformation compared with 0.51% to *M. leprae*.

The cross-reactivity between BCG and *M. leprae* is a matter of special concern because of the efforts to establish an effective immunoprophylaxis in leprosy. Still a matter of dispute due to the conflicting results in the Uganda (5) and Burma (3) trials, the effect of BCG vaccination in the prevention of leprosy awaits further clarification.

In the present investigation an attempt is made to use the LTT technique to study the antigenic cross-reactivity between BCG and *M. leprae*. It compares the reaction of a group of healthy Norwegians to BCG and *M. leprae* in the LTT. Because BCG vaccination is obligatory in Norway, it was decided to perform the study on two groups of BCG-vaccinated subjects, one tuberculin positive and the other tuberculin negative, as defined by skin testing.

**MATERIALS AND METHODS**

**Test subjects.** Seven tuberculin-positive and seven tuberculin-negative individuals were included in the study. In the tuberculin-positive group the mean age was 25.0 years (range, 20 to 35), and in the tuberculin negative it was 27.4 years (range, 19 to 37). Both groups consisted of one male and six females and, with one exception, were all previously BCG vaccinated, vaccinations having been carried out more than 6 months previously. All the test subjects were working at Ullevål Hospital, Oslo.

**Skin testing.** All subjects were initially tested by the von Pirquet scarification test using old tuberculin (Veterinary Institute, Oslo, Norway) as the antigen. The reaction was read after 72 h. Those included in the tuberculin-positive group all had an induration measuring 10 mm or more. Those who were completely negative in this test were retested by injecting 0.1 mg of old tuberculin intradermally. Those showing an induration measuring less than 4 mm 72 h later were regarded as tuberculin negative.

**Purification of lymphocytes.** Venous blood (30
ml) was drawn with a heparinized, disposable plastic syringe using 20 IU of heparin per ml of blood (heparin without preservative; Ullevål Apotek, Oslo, Norway). The method of Bøyum (4) was used with slight modifications. The blood was mixed with an equal amount of 0.9% NaCl and then layered on top of 20 ml of Ficoll/Isopaque, density 1.077 (10 parts of 33.9% Isopaque [Nyegaard & Co. A/S, Oslo]) plus 24 parts of 9% Ficoll [Pharmacia AB, Uppsala, Sweden]), in a 100-ml centrifuge tube. The tube was centrifuged in a swing-out rotor for 45 min at a speed giving 300 × g at the interphase between the two fluid layers. The cells that accumulated at the interphase were carefully collected with a Pasteur pipette. They were then mixed with 2 volumes of Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y. [GIBCO]) containing 2.5 IU of heparin per ml and centrifuged for 10 min at 250 × g. The cells were washed two times in Hanks balanced salt solution and finally resuspended in medium TC 199 (GIBCO) with 20% human AB serum at a concentration of 10⁷ cells/ml.

Cultures of lymphocytes. The cells were cultured in plastic trays for tissue culture (Linbro IS-FB-96-TC, Linbro Chemical Co., New Haven Conn.). The culture medium consisted of TC 199 with Earles salts (GIBCO), containing 20% human AB serum, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml (Difco Laboratories, Detroit, Mich.). The cultures were incubated at 37 °C in an atmosphere containing 5% CO₂ and 70 to 80% relative humidity. Mitogen-stimulated cultures were harvested on day 3, whereas antigen-stimulated cultures were harvested on day 6. Pilot studies had shown that when an optimal concentration of stimulant was used peak response was obtained at those times.

Stimulation of lymphocyte cultures. Ten microliters of the stimulating substance was added per culture. The following substances were used. Phytohemagglutinin HA15 (Wellcome Reagents Limited, Beckenham, England) was dried, reconstituted by adding 5 ml of distilled water, and stored at −20 °C until used. Ordinary purified protein derivative (PPD) tuberculin and PPD tuberculin made from BCG (PPD/BCG) (The Veterinary Institute, Oslo, Norway) were stored as concentrated solutions at 4 °C. M. leprae prepared from human skin biopsy material was kindly provided by T. Godal, Armauer Hansen Research Institute, Addis Ababa, Ethiopia. It was stored at −20 °C, and some of the bacilli were frozen and thawed several times before use. M. tuberculosis bouis strain BCG (BCG), obtained as lyophilized vaccine (BCG Laboratoriet, Bergen, Norway), was resuspended in saline and kept at −20 °C until used. Antigenic extract from Candida albicans (lot no. L 54558, Hollister-Stier Laboratories, Spokane, Wash.) was stored at 4 °C. All stimulated and control cultures were set up in triplicate.

Radioactive labeling. Tritiated thymidine with a specific activity of 2 Ci/mM was added to the cultures 16 to 18 h before harvest. The resulting concentration of radioactivity was 2 μCi/ml of culture medium.

Harvesting of cultures. When the cultures were terminated, the trays were either harvested immediately or frozen and kept at −20 °C for some days. By using a specially constructed harvesting device, the cultures were transferred by suction, six at a time, onto glass-fiber filters (Gelman type A, Gelman Instrument Co., Ann Arbor, Mich.) 45 mm in diameter. Each well was then washed through with distilled water to complete the transfer of cells to the filters. Subsequently each filter was washed with 20 to 25 ml of distilled water and finally with 5 to 10 ml of 96% ethanol. The filters were transferred to counting vials and dried at 60 °C for 1 h.

Liquid scintillation counting. A 5-ml amount of toluene-based scintillator [5 g of 2,5-diphenyloxazole and 0.15 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene] was added to each counting vial before counting in an Intertechnique SL31 liquid scintillation counter (Intertechnique, Plaisir, France).

Statistical methods. Thymidine incorporation in counts per minute was calculated as the arithmetic mean of triplicates. Wilcoxon’s sign rank test (23) was used to test differences between groups. The correlation coefficient r and the regression coefficient b were calculated with a Wang 720 A/B electronic computer (Wang Laboratories, Inc. Tewkesbury, Mass.) equipped with a program for linear regression. Values of P below 0.05 were regarded as statistically significant.

RESULTS

Lymphocytes from tuberculin-positive and tuberculin-negative subjects, hereafter referred to as positive and negative, respectively, were stimulated in vitro with four different concentrations of PPD tuberculin. As far as possible, lymphocytes from one positive and one negative donor were prepared on the same day. The results are shown in Fig. 1A. There was a statistically significant difference between the positive and negative groups at all four concentrations. However, the difference between the two groups became less when high concentrations (40 μg/ml) were used, because such high concentrations of PPD caused considerable stimulation in the negative group.

Tuberculin prepared from the BCG strain of M. tuberculosis bouis stimulated the cultures somewhat less than ordinary PPD tuberculin per microgram of protein (Fig. 1B). Again, lymphocytes from positive subjects responded more strongly than lymphocytes from negative subjects. This difference was statistically significant in the dose range 0.4 to 40 μg/ml. At high concentrations (40 μg/ml) lymphocytes from negative individuals were stimulated less than by ordinary PPD.

As a third specific stimulus whole BCG bacilli were used (Fig. 2). In the dose range 3 × 10⁴ to 3 × 10⁷ bacilli/culture, the degree of stimulation was only moderately influenced by the number of bacilli added. Optimal stimulation was obtained by adding 3 × 10⁴ bacilli/culture, whereas an antigen dose 10 times higher
caused depression of the response. All four doses of antigen induced a significantly higher response in the positive than in the negative group ($P < 0.005$). It was concluded from these experiments that lymphocytes from tuberculin-positive subjects responded more strongly than lymphocytes from tuberculin-negative subjects against all three mycobacterial antigens. Furthermore, when regarding lymphocyte cultures from separate donors the responses to these antigens were found to be closely correlated; e.g., between $3 \times 10^4$ BCG and 4.0 $\mu$g of PPD per ml, the correlation coefficient was 0.81.

Lymphocytes from the two groups of individuals were also exposed in vitro to leprosy bacilli. The response to two different preparations of M. leprae, ML2 and ML4, is shown in Fig. 3. Again, the response was strongest in the positive group; with $10^8$ bacilli added per culture the difference was statistically significant ($P < 0.01$).

The finding that lymphocytes from the positive group responded more strongly to M. leprae might be due to an increased responsiveness of these cells to in vitro stimuli in general. Therefore, lymphocytes from all individuals of the two groups of donors were also exposed to phytohemagglutinin, which is a nonspecific stimulant, and to an extract from C. albicans, which is a species without any known antigenic relationship to mycobacteria. It was found that...
lymphocytes from the positive and the negative groups did not differ significantly in their response to these stimulants.

The responses to \textit{M. leprae} and BCG were compared quantitatively to obtain information on the cross-reactivity between these mycobacteria in a system measuring cell-mediated immunity. Table 1 shows the increase in thymidine uptake by lymphocytes from tuberculin-positive donors after exposure to BCG and \textit{M. leprae}. It is seen that the response to \(10^6 \text{ M. leprae}\) is between 9.4 and 47.8\% of the response to \(3 \times 10^4 \text{ BCG}\), whereas it amounts to 23 to 89.2\% of the response to \(3 \times 10^4 \text{ BCG}\). These data indicate that a certain cross-reactivity exists between \textit{M. leprae} and BCG in the LTT. However, the degree of antigenic similarity between the two mycobacteria is not easily assessed on the basis of such data.

The lymphocyte response to in vitro stimulation with antigen is known to be dose dependent; optimal stimulation is only achieved within a certain dose range and both higher and lower concentrations of antigen result in lower stimulation. If the LTT is used to quantitate cross-reactivity, it is therefore important to use the antigens which are to be compared in equal concentrations. The difficulties which may arise when comparing the responses to antigens that are mixtures of several components are illustrated in Fig. 4. In this figure the responses to equal protein concentrations of PPD and PPD/BCG are compared. At 0.4 \(\mu\)g/ml the response to PPD/BCG was much weaker than the response to PPD, and the correlation coefficient was only 0.16 (Fig. 4A). At 4 \(\mu\)g/ml the two responses were more equal (\(b = 1.67\)) and nearly linearly correlated (\(r = 0.96\)) (Fig. 4B), whereas at 40 \(\mu\)g/ml the two responses correlated poorly (\(r = 0.36\)) (Fig. 4C). Apparently the PPD preparation was more potent per microgram of protein; the response to 0.4 \(\mu\)g of PPD per ml was almost equal to the response to 4.0 \(\mu\)g of PPD/BCG per ml (\(b = 1.25; r = 0.98\)). The high correlation coefficient and the regression coefficient, being near to 1.0, indicate that the antigens which induce lymphocyte transformation are nearly identical in the two preparations.

Figure 5 compares the response to BCG and \textit{M. leprae} in the LTT. As expected the structural similarity between these two antigens appears to be less than between PPD and PPD/BCG.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Control</th>
<th>A ((3 \times 10^4 \text{ BCG}))</th>
<th>B ((3 \times 10^4 \text{ BCG}))</th>
<th>((10^6 \text{ M. leprae}))</th>
<th>C as % of A and B</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN</td>
<td>3,160 ± 570</td>
<td>40,830 ± 3,420</td>
<td>67,280 ± 2,180</td>
<td>12,690 ± 1,210</td>
<td>31.0-18.8</td>
</tr>
<tr>
<td>BT</td>
<td>630 ± 80</td>
<td>6,020 ± 1,130</td>
<td>14,650 ± 800</td>
<td>1,390 ± 190</td>
<td>23.0-9.4</td>
</tr>
<tr>
<td>AMS</td>
<td>490 ± 60</td>
<td>2,330 ± 180</td>
<td>5,810 ± 400</td>
<td>1,200 ± 340</td>
<td>51.5-20.6</td>
</tr>
<tr>
<td>RJN</td>
<td>1,180 ± 310</td>
<td>4,310 ± 670</td>
<td>6,840 ± 970</td>
<td>2,200 ± 320</td>
<td>51.0-32.1</td>
</tr>
<tr>
<td>EG</td>
<td>1,920 ± 270</td>
<td>9,010 ± 1,510</td>
<td>20,990 ± 1,200</td>
<td>8,040 ± 1,330</td>
<td>89.2-38.3</td>
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<tr>
<td>OC</td>
<td>250 ± 50</td>
<td>6,390 ± 440</td>
<td>8,220 ± 1,500</td>
<td>3,930 ± 320</td>
<td>61.5-10.4</td>
</tr>
<tr>
<td>RN</td>
<td>550 ± 50</td>
<td>5,850 ± 610</td>
<td>16,030 ± 1,150</td>
<td>1,670 ± 540</td>
<td>28.5-10.4</td>
</tr>
</tbody>
</table>

\[\text{*In columns A, B, and C, counts per minute} = \text{counts per minute in stimulated cultures minus counts per minute in control cultures. SE, Standard error of the mean.}\]

**Fig. 4.** Lymphocyte response in vitro to stimulation with various concentrations of PPD tuberculin and PPD tuberculin made from BCG (PPD/BCG). (A) 0.4 \(\mu\)g/ml; (B) 4.0 \(\mu\)g/ml; (C) 40 \(\mu\)g/ml. Symbols are as in Fig. 1.
BCG. The correlation coefficient is 0.59, which is statistically significant at the 5% level (23), and the regression coefficient is 0.29.

**DISCUSSION**

Delayed skin reactions and lymphocyte stimulation in vitro both depend on the presence of sensitized lymphocytes in the circulation. A high degree of correlation is therefore expected between these two tests. There is, however, conflicting evidence on this point. Concerning tuberculin, which is a classic antigen in delayed hypersensitivity, some authors have been able to demonstrate good correlation between in vitro tests and skin testing (14, 15, 18) whereas others have found no such correlation (1, 16, 27). These discrepancies may be due to variations in experimental technique, e.g., with regard to PPD concentrations used and timing of the measurements of lymphocyte reactivity (18).

In the present study a clear correlation was found between the result of skin testing with tuberculin and the lymphocyte response in vitro against PPD, PPD/BCG, and whole BCG bacilli. It is important to note that in the in vitro system the difference between the tuberculin-positive and the tuberculin-negative groups was not the same at all concentrations of antigen. This was seen most clearly with PPD, which in high concentrations induced transformation in both groups, possibly due to nonspecific mitogenic activity of PPD (26). The in vitro response to high doses of this antigen therefore was less clearly related to skin test responses than the response to lower doses. At optimal concentrations of antigen the separation of the responses in the two groups and the ratio between them was similar with all three antigens. When the responses to the three antigens were compared for each individual, a high degree of correlation was also found, indicating that the response is mainly directed against antigens which are present in all three preparations.

In tuberculosis the degree of systemic cell-mediated immunity to tuberculin does not seem to correlate well with the degree of protection against infection. Individuals who are tuberculin negative after adequate BCG vaccination have obtained the same degree of protection against tuberculosis as those who become tuberculin positive (17). It has been assumed that this indicates that PPD does not contain the most important antigens related to immunity against tuberculosis (9). If our tuberculin-negative group has the same degree of protection against tuberculosis as the tuberculin-positive group, this is not revealed any better using whole BCG bacilli as the antigen.

At low antigen concentrations the potency of the three preparations was clearly different. The high efficiency of BCG as a stimulant in vitro may be due to the physical nature of the antigen; at low concentrations PPD bound to particles has been found to induce much stronger responses than soluble PPD (20). Therefore, it was considered correct to use whole BCG bacilli as the reference antigen when evaluating the response to M. leprae.

Due to a more limited supply of antigen the dose response relationship for M. leprae could not be fully established. However, with the highest concentration of M. leprae the lymphocyte response was significantly higher in the tuberculin-positive than in the tuberculin-negative group. Furthermore, the response to phytohemagglutinin and Candida antigen was similar in both groups. The most probable explanation for these findings is that BCG vaccination has induced an immune response against antigens that are present both on BCG and on M. lepra. It seems much less likely that the reaction to M. lepra is due to immunization with mycobacteria other than BCG, present in the environment. The lack of reaction in the negative group would then be explained by assuming that these individuals respond more weakly to mycobacterial antigens in general.

In serological systems extensive cross-reactions occur between various mycobacterial species (24). Furthermore, antibodies against more than one-half of the BCG antigens detectable by crossed immunoelectrophoresis were found in sera from patients with lepromatous leprosy (2). Several studies have shown that the cross-reactivity against molecules or cells carrying common determinants is more pronounced in
T-cell than in B-cell responses (12). Therefore, it seems likely that cross-reactivity exists between *M. leprae* and BCG also in cell-mediated immune responses.

In its present modification the LTT appeared to be less specific than previously reported; the response to *M. leprae* was on the order of 20 to 50% of the response to BCG, whereas earlier estimates have been as low as 7% (10). This discrepancy is probably due to different sensitivity of the methods used. Clearly, a relatively low sensitivity is an advantage when the LTT is used in diagnostic and epidemiological work to reduce the number of "false-positive" reactions. Conversely, if it is used to study the antigenic relationship between two microorganisms, it should have a high sensitivity. In any instance the degree of cross-reactivity found is intimately related to the method used.

Some of the problems which may arise when LTT is used to evaluate cross-reactivity in complex systems have been illustrated with data concerning the two antigens PPD and PPD/BCG. In tuberculin, which is a mixture of several components, the concentration of substances which induce transformation cannot be determined by measuring the protein concentration. Thus, although the two tuberculins were used in the same protein concentrations, the PPD preparation was found to be a more potent in vitro stimulant than PPD/BCG. Similar variations in potency have been observed between different batches of the same PPD tuberculin (19). When evaluated by skin testing 3 and 6 months after BCG vaccination, the reaction to PPD/BCG has been found to be somewhat less than the reaction to ordinary PPD, but otherwise the two parallel each other closely (25). The present results accord well with these findings, provided comparison is based on the responses to 4.0 µg/ml; at higher or lower concentrations the responses to the two tuberculins are less closely correlated (see Fig. 4).

When tested against lymphocytes from various donors, the responses to two antigens will parallel each other to the extent that the antigens are structurally related, and their relationship is reflected by the correlation coefficient and the regression coefficient. If the antigens are not pure substances but mixtures of several components present in unknown quantities, it is difficult to ensure that the stimulating doses are commensurable. Since the correlation coefficient r is expected not to be influenced by antigen dose as long as both antigens induce specific stimulation, r appears to be a better estimator of cross-reactivity than the regression coefficient. However, apart from the fact that a high value of r may indicate that two antigens are identical, as in the case of PPD and PPD/BCG, the correlation coefficient does not allow direct quantification of cross-reactivity, and the antigenic similarity between BCG and *M. leprae* therefore cannot be quantified by correlating the LTT responses. Further progress in this field requires work with the relevant antigens in purified form.

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LITERATURE CITED


LYMPHOCYTE RESPONSE TO PPD, BCG, AND M. LEPRAE